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HISTOFLUORESCENT LOCALIZATION OF SEROTONIN AND DOPAMINE IN THE NERVOUS SYSTEM AND GILL OF *MYTILUS EDULIS* (BIVALVIA) [1]

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In bivalve molluscs there has been physiological evidence of neural control of ciliary activity (Koshtoyants, Buznikov and Manukhin, 1961; Aiello and Guideri, 1964, 1966; Takahashi and Murakami, 1968). There has also been histological evidence for the innervation of ciliated gill filaments (Splittstosser, 1913; Setna, 1930; Aiello and Guideri, 1965; Sweeney, 1968). Specifically, in *Mytilus edulis*, Aiello and Guideri (1965) have reported that nerve fibers from the branchial nerve enter individual gill filaments and run beneath the lateral cells. An electron micrographic description of this innervation has been given by Paparo (1972). Sweeney (1968) has reported the presence of nerves in the gill filament of *Sphaerium sulcatum*. Grave and Schmitt (1925) reported a bipolar nerve cell system beneath the ciliated epithelium of the gill in the bivalves *Lampsilis*, *Anodonta* and *Mya*.

It has also been established in *M. edulis* that transection of the branchial nerve depresses ciliary activity and this activity was increased by electrical stimulation in the intact animal or in the gill-nerve-visceral ganglion preparation (Aiello and Guideri, 1964, 1965; Takahashi and Murakami, 1968).

Other experimenters have proposed the concept of local hormonal control employing acetylcholine (Bulbring, Burn and Shelly, 1953) or serotonin (Gosselin, Moore and Milton, 1962). Acetylcholine has been found to be both a positive and a negative modulator of the frontal cilia and this effect is concentration dependent (Bulbring *et al.*, 1953; Aiello and Paparo, 1974). However, acetylcholine esterase activity is largely localized in the nervous system (Bouffard, 1970) and the response of cilia on the gill to electrical stimulation of the branchial nerve is altered by the administration of physostigmine, a cholinesterase inhibitor (Aiello and Paparo, 1974). One might equally well, therefore, attribute to endogenous acetylcholine the role of neurotransmitter, with possible actions both on ciliated cells and on elements of the nervous system.

Serotonin was also found to have a cilio-excitatory and metabolic stimulatory effect in several lamellibranch gills, and may be an endogenous regulator (Gosselin *et al.*, 1962; Schor, 1965; Sweeney, 1968; Aiello, 1970). Its precursors, tryptophan and 5-hydroxytryptophan have been found in the gill of *M. edulis* (Aiello, 1960, 1962; Gosselin *et al.*, 1962), and the conversion of 5-hydroxytryptophan to serotonin and further breakdown products has been demonstrated (Blaschko and Milton, 1960; Aiello, 1965). It is not known exactly where these substances are located but Sweeney (1968) has demonstrated the occurrence of serotonin

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and some catecholamine in gill filaments of *S. sulcatum* by the histochemical fluorescence technique.

A second monoamine implicated in the regulation of ciliary activity is dopamine. The administration of dopamine to the lateral cilia results in a cilioinhibition (Paparo and Aiello, 1970). Dopamine has been found in the nervous system of a number of bivalve molluscs (Dahl, Falck, Lindquist and Von Mecklenburg, 1962; Dahl, Falck, Von Mecklenburg, Myhrberg and Rosengren, 1966; Sweeney, 1963, 1968) and in extracts of bivalve gills (Malanga, Wenger and Aiello, 1972).

In *M. edulis* the only report of ganglionic morphology was published in 1887 by Rawitz. He concluded that histologically the ganglia of *M. edulis* follow the typical pattern for Pelecypods, having a cortex surrounding a central neuropile and containing no large cell bodies or fibers (Rawitz, 1887). Localization of monoamines in invertebrates became possible with the advent of the histochemical fluorescent technique of Falck, Hillarp, Thieme and Torp (1962). Various investigators have used this technique successfully in bivalves (Dahl *et al.*, 1966; Zs.-Nagy, 1968; Sweeney, 1968).

The purpose of this research was to see if the localization of the monoamines serotonin and dopamine is correlated with the control of ciliary activity. The physiological and pharmacological evidence cited above seems to call for the dual innervation of the ciliated epithelium of *M. edulis* by serotonin and dopamine containing neurons. It was also of interest to know more about the central nervous system itself such as the distribution and interrelationship of specific neurons in the ganglia and the location of cell bodies of neurons innervating the gill in the hope of being able to relate this information to an understanding of ciliary control. A preliminary report of some of this work has already been presented (Stefano and Aiello, 1974).

Materials and Methods

Mytilus edulis was obtained from two sources: some animals were harvested from rocks in the intertidal zone of Pelham Bay, Long Island Sound, New York and some animals were obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts. The animals were kept in artificial seawater (Instant Ocean Aquarium System) at 19° C, pH 7.5–7.8 and specific gravity 1.025.

Three criteria were used in selecting a healthy animal for experimentation: (1) it closed its shell quickly when the siphon was touched; (2) when the foot in the gaping animal was touched, it reflexively withdrew it and then closed the valves; (3) the valves remained closed against a reasonable amount of force applied to open them.

Treatment of whole animals with chemical agents was accomplished either by injection or by direct exposure in the bathing medium. For injection, the valves were opened with a retractor and injection made into the foot or the posterior adductor muscle and sometimes in both. The animal was then placed in a liter beaker with 300 milliliters of seawater and its own oxygen source, but maintained at the original temperature by immersing the beaker in the main aquarium. The same system was utilized for direct exposure, except that the chemical agent was dissolved in the bathing medium.

The valves were forced open and the posterior adductor muscle cut midway

between the left and right visceral ganglia. The cerebral ganglia were removed first. This was done by placing a relatively thick piece of paper under them and pinning tissue around the ganglia to the paper. Then the tissue and paper was cut free and immersed in isopentane cooled by liquid nitrogen to about −170° C. The visceral ganglion was removed with a piece of supporting posterior adductor muscle and frozen as above. In some instances the visceral ganglion and associated gill were removed together for further experimentation.

Frozen tissues were transferred rapidly to the thermal plate of an Edwards-Pearse Tissue Freeze Dryer and dried for five days at a temperature of −45° C and a vacuum of 10^{-3} torr.

After this drying period the tissues were gradually brought to room temperature and the vacuum broken by the introduction of dry nitrogen into the chamber. The tissues were rapidly transferred to a steel grid over dry paraformaldehyde in a small, wide-mouthed jar. The paraformaldehyde was routinely stored *in vacuo* over phosphorus pentoxide at room temperature. This jar was then sealed by a screw cap, placed in preheated oven and kept at a temperature of 80° C for 1.5 hrs.

Paraffin to be used for embedding was melted at 56° C and degassed in a vacuum. Small amounts were further degassed in a container on the thermal plate of the tissue drier and allowed to cool. A pit was dug into the wax and the dry, paraformaldehyde-treated tissue was placed in the pit. The tissue was infiltrated in a vacuum at 60° C for approximately one hour or until bubbles ceased to appear from the tissue, which was never more than two hours. The vacuum was then broken and the tissue transferred to fresh paraffin for about four hours at 60° C, then placed in plastic embedding blocks and solidified at room temperature.

Tissues were sectioned on a standard microtome. The sections were mounted on non-fluorescent slides in Fluoromount (Fisher) containing 10% dry xylene or in dry xylene alone and flattened on a hot plate at 32° C.

Fluorescence was observed with a Reichert Zetopan fluorescent microscope fitted with Mercury Lamp HBO 200 W and either bright field or dark field Reichert condensors. The excitation filters BG-12 permitted activation at 410 nm to 440 nm, and UV-blue excluding filters GG9 and GG1 permitted the passage of green fluorescence from catecholamine and yellow fluorescence from serotonin reaction products (Marsden and Kerkut, 1969; Falck *et al.*, 1962; Falck and Owman, 1965). After observing fluorescence the slide was left in place and the optics changed to phase contrast as an aid in identifying the fluorescent structures. Some sections were stained with Harris hematoxylin and eosin. Other tissues were treated with Ramon y Cajal's silver-pyridine method (Favorsky, 1930).

A single lens reflex camera (Minolta SRT 101) was utilized for photomicroscopy. Color photography was done with High Speed Ektachrome film (ASA 164) and processed commercially. Black and white photography was done with Tri-x film (ASA 400) developed in Kodak D76 and printed on Kodak Polycontrast F or Kodabromide F-2 paper.

Autofluorescence was differentiated from specific fluorescence by comparing paraformaldehyde treated tissue to untreated tissue; fluorescence common to both was termed autofluorescence. Yellow autofluorescence was further differentiated

from specific serotonin fluorescence in that the fluorophore of serotonin fades rapidly while autofluorescence remains. A specific test developed by Corrodi, Hillarp and Jonsson (1964) was also used in which the section on the slide was partially hydrated and treated with 0.03% sodium borohydride for two minutes and then re-examined under the fluorescence microscope. Autofluorescence remained but the specific fluorescence was reduced by sodium borohydride to a non-fluorescent product. Specific fluorescence was regained by resubjecting the slide to the paraformaldehyde treatment.

Observation and Results

Gross anatomy

The structures in the nervous system of *Mytilus edulis* examined in this investigation were the cerebral and visceral ganglia, the cerebrovisceral connective and the branchial nerve and its subdivisions. Our description is in essential agreement with that of Field (1922) except that he could not trace nerve fibers from the branchial nerve into the individual gill filaments. All indications are that *M. edulis* is bilaterally symmetrical throughout the animal and that our descriptions apply equally well to either side.

The cerebral ganglia are narrow bodies with their long apices pointing posteriorly. They lie on the ventral side of the esophagus 4 to 6 mm apart and united by a commissure. Each ganglion is about 2 mm in length and 1 mm wide at its base. The thickness varied but appeared to be approximately 0.5 mm at most. In some specimens, there was an orange-red pigment on its outer ventral surface of each ganglion.

The posterior trunk of each cerebral ganglion passes backward and outward across the ventral side of the anterior retractor muscle. At the lateral side of the anterior retractor muscle the trunk divides into two separate components, the cerebrovisceral and the cerebropedal connectives. The cerebrovisceral connective turns upward and continues in a posterior direction traveling along the lateral surfaces of the posterior retractor muscles and terminating in the visceral ganglion on that side.

The visceral ganglia are situated on the anterior ventral surface of the posterior adductor muscle close to the area where the gills are suspended. Each ganglion gives rise to several nerves. The branchial nerve leaves the posterior lateral side, runs obliquely ventrally and posteriorly to the base of the gill and sends a large number of fine fibers into the gill axis on that side of the animal.

Histology, cells of the cortex

Tissues stained by the standard hematoxylin and eosin procedure revealed the typical structural organization of the ganglia (Fig. 1). The largest nerve cell bodies were approximately 15 μ in diameter but the majority tended to be about 9–11 μ in diameter. There were also various types of smaller cells situated around the larger ones. The nerve cell bodies in both the cerebral and visceral ganglia were found to contain a granular cytoplasm with many inclusions, the largest of which were approximately 1 μ in diameter. The nerve cells were almost exclusively unipolar, having an approximately spherical cell body and a single grad-

ually tapering cell process. Through the use of Ramon y Cajal's silver-pyridine method, which stained fine fibers, varicosities, and endings with a darker intensity, the cortex was found to be an area rich in terminal varicosities.

In general, the cells of the cortex appeared to be concentrated toward the ganglion's ventral surface. They were not uniformly distributed concentrically within the cortex, but were grouped into clusters.

Neuropile region

In both the visceral and cerebral ganglia a few nerve cell bodies were found in the neuropile region. These nerve cell bodies appeared to be extensively innervated as indicated by the specific staining of endings on their surface. The silver-pyridine method clearly showed how the process of the nerve cell body tapered as they extended from the body. The neuropile region offered a tangled appearance so that following an individual fiber for more than 15 μ was impossible. The fibers in the neuropile, however, did appear to possess some organization as shown by the presence of nerve tracts.

Connective tissue sheath

The connective tissue sheath in *M. edulis* not only covered the ganglia but also the connectives and nerves which emanated from the ganglia. Nerve cell bodies of the ganglia continued into the beginnings of the nerves originating from the ganglia, and these were found to be especially close to the sheath. By the use of the silver-pyridine method, nerve fibers were shown to be present in the sheath itself. No muscle fibers were observed there.

Fluorescent structures in the cerebral ganglion

In the cerebral ganglion specific yellow fluorescence was found in nerve cell bodies of 9 to 14 μ diameters located in the cortex (Fig. 2). Many of these cells were radially oriented and closely associated with the connective tissue sheath but a few were scattered throughout the cortex. The sheath had within itself yellow fluorescing granules, most of which seemed to be connected by fine yellow fibers, thereby constituting a network of varicose fibers (Fig. 2). Many of the nerve cell bodies in the ganglion cortex did not fluoresce (Fig. 2). In the neuropile region of the cerebral ganglion there were no yellow fluorescing cell bodies, but there were yellow granules, which gave the appearance of beads or varicose fibers. The distinction between granules and cell bodies had to be made under a 100 ×, oil immersion lens. There were also fine, yellow, varicose fibers in both the neuropile and the cortex.

The cerebral ganglion also contained green fluorescing nerve cell bodies (Fig. 3) which for the most part were slightly smaller in diameter than those emitting yellow fluorescence (Fig. 2). They were found in the cortex but not as close to the connective tissue sheath as the yellow fluorescing cells. The green fluorescence was not restricted to small intracellular granules but appeared to be homogeneous in its distribution (Fig. 3). The neuropile was extremely rich in green fluorescing fibers.

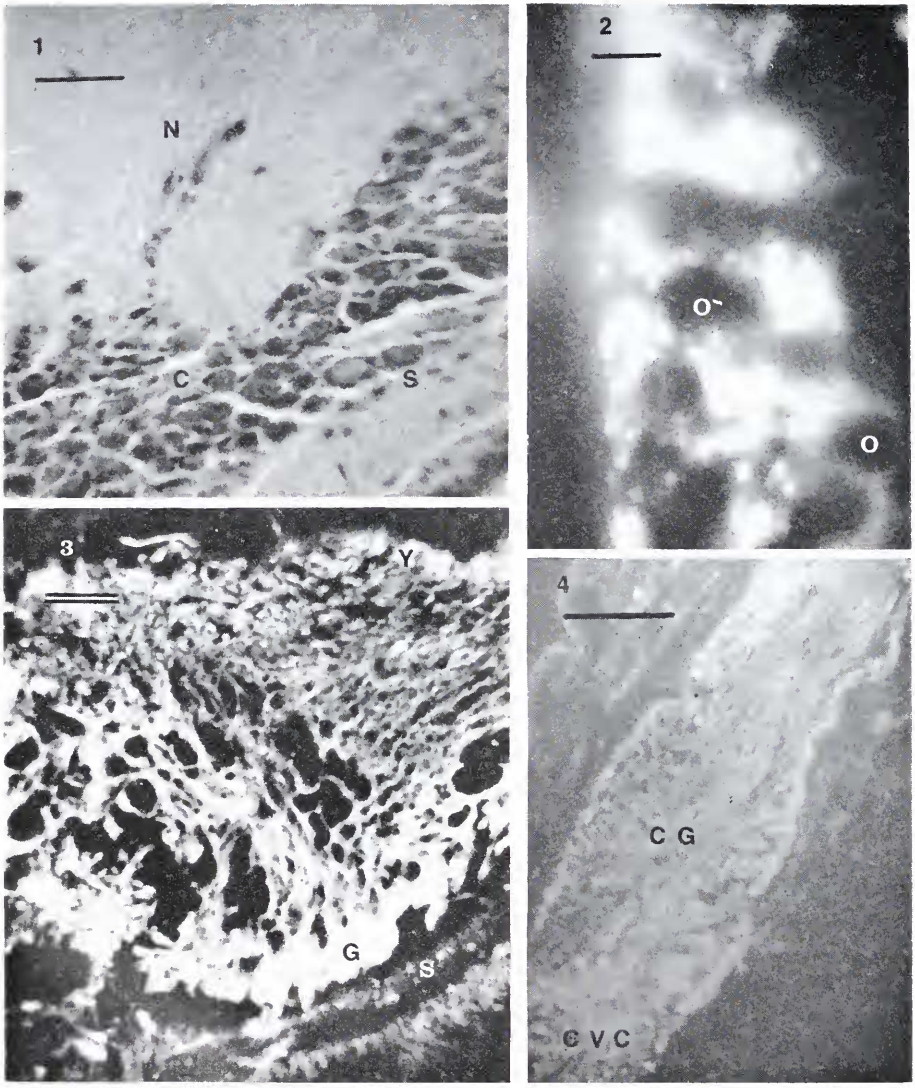

FIGURE 1. Hematoxylin and eosin stained section, 7 μ thick, of the cerebral ganglion showing representative appearance of neuropile (N), cortex (C) and thin connective tissue sheath (S). Scale bar = 10 μ.

FIGURE 2. Fluorescent photomicrograph of outer-part of the sheath and cortex of the cerebral ganglion showing cells packed with yellow fluorescing granules interspersed with non-fluorescing cells (O); (cells were identified by phase contract microscopy of same preparation). The sheath contains yellow fluorescing fibers and granules of similar appearance but no cells. Scale bar = 5 μ.

FIGURE 3. Cross section of visceral ganglion showing groups of green fluorescing cells (G) clustered at the periphery, especially on the ventral side (bottom of picture). High magnification never reveals discrete granules, as with yellow fluorescing cells (Y), but homogeneous green fluorescence. A few granules and fibers of the neuropile are yellow, the

Fluorescent structure in the visceral ganglion

The visceral ganglion contained only a few yellow fluorescing nerve cell bodies, these being 11 to 14 μ in diameter. Some yellow fibers were associated with both the cerebrovisceral connective and the branchial nerve. Yellow fibers were also seen in the predominantly green cortical region and neuropile along with yellow fluorescent beads previously described. The majority of the beads were present in the neuropile, while only a few were in the cortex.

The visceral ganglion cortex contained many green fluorescing cell bodies (Fig. 4) most of which were 10–12 μ in diameter and exhibited a homogenous green fluorescence. Processes could be seen to emanate from the nerve cell bodies. Green fibers could be seen in the cerebrovisceral connective and in the origin of the branchial nerve.

Also present in the visceral ganglion, especially in summer-harvested animals, was an orange auto-fluorescent pigment. This pigmented material was highly clustered in one area of the visceral ganglia and very little was found outside this area. The pigmented cells were glandular in appearance, no processes were observed. In summer animals, this pigment was in the cerebral ganglia also. Some orange fluorescing pigment was also found intracellularly within the nerve cell bodies.

Fluorescent structures in peripheral nerve

The branchial nerve contains both yellow and green fluorescing fibers as well as the yellow bead-like structures mentioned earlier. Along its length, the branchial nerve gives off many branches which run in the gill axis. One such branch could be traced in the gill axis and was seen to have further divisions leading into the individual gill filaments (Fig. 5). The branches entering the gill filament could be followed for some length, but once coming into the vicinity of the supporting rod, they would appear to merge with it. The fluorescence of these branches of the branchial nerve and elements of it that enter the gill filament appeared to be made up of green and yellow fibers. It was difficult to tell whether there was a mixing of the amines in one nerve fiber or there were two types of fibers, each emitting its own fluorescence. Essentially what was occurring was a kind of masking effect which prevented the resolution of individual fibers and colors.

The supporting rod within the individual gill filaments exhibited specific green fluorescence which disappeared with sodium borohydride treatment. The epithelial cells in the gill filament had no specific fluorescence. Nucleated blood cells were found not only within the blood sinus but between epithelial cells of the gill filament. These blood cells exhibited a specific yellow fluorescence originating from granules within their cytoplasm and could be clearly identified as blood

majority are green and nonfluorescent. The sheath (S) has both yellow and green fluorescing granules and fibers. Scale bar = 50 μ.

FIGURE 4. Longitudinal section through the cerebral ganglion (CG) seven days after cutting the cerebrovisceral connective (CVC). The fluorescence is intensely yellow with no detectable green. High magnification fails to reveal the discrete yellow fluorescing granules seen in the control animals; the cells now being filled with intensely fluorescent material. Scale bar = 50 μ.

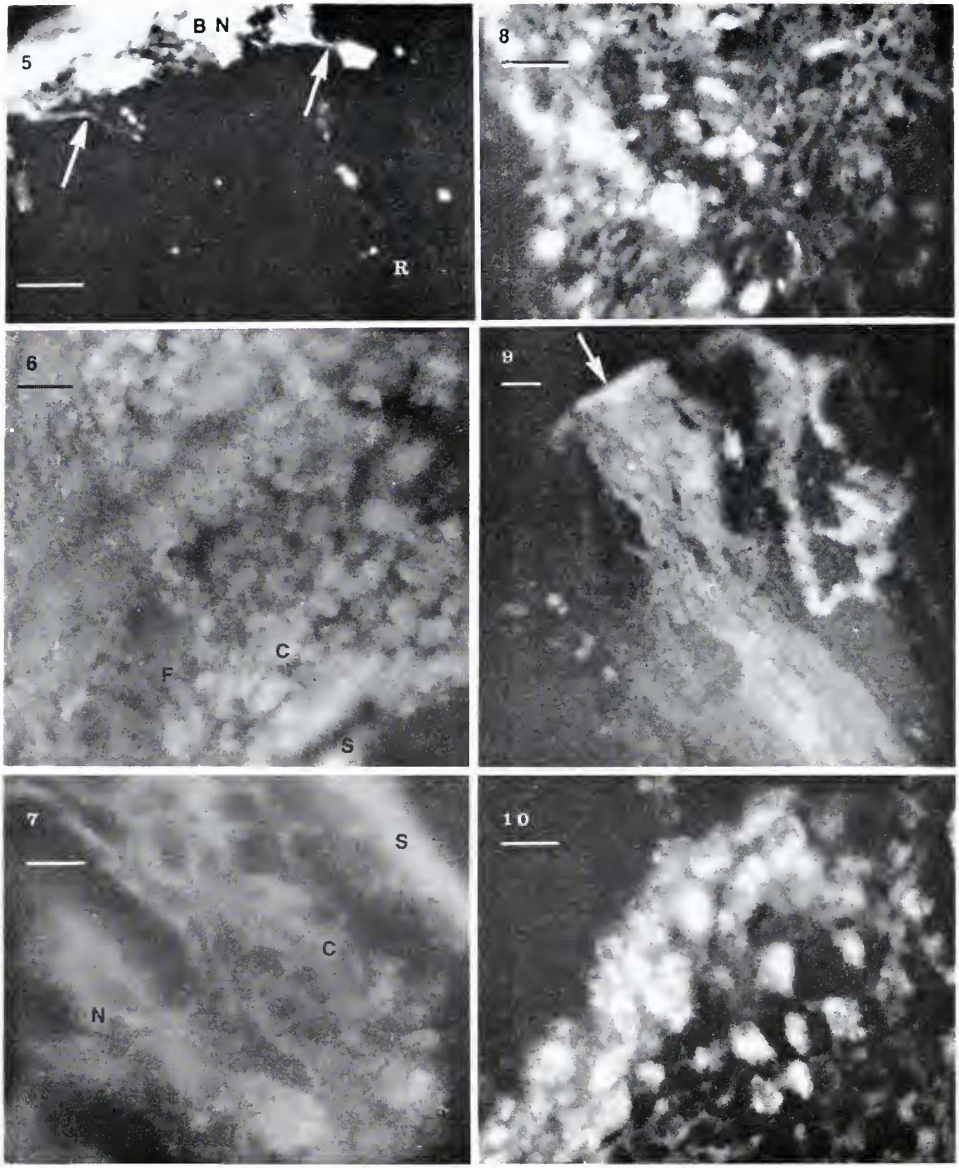

FIGURE 5. A fluorescent photomicrograph of a section through the gill axis at the origin of several filaments. A branch of the branchial nerve (BN) gives off one or more individual fibers (arrows) to each filament. Within the filament the fibers are weakly visible against the faintly green supporting rod (R). Scale bar = 50 μ.

FIGURE 6. Section of cerebral ganglion two days after the intramuscular injection of serotonin. All cells (C) and fibers (F) and the sheath (S) fluoresce yellow, indicating the relatively indiscriminate uptake of the amine. Scale bar = 20 μ.

FIGURE 7. Section of cerebral ganglion from reserpinized mussel. Despite low level of green-yellow fluorescence prolonged photographic exposure reveals an indiscriminate distri-

cells by their appearance, location, and lack of processes or attachment to any other cell.

Denervation experiments

Animals used for these experiments had their valves held open with a retractor. The cerebrovisceral connective on one side was cut about 0.5 cm from the visceral ganglion. After this operation, the animals were placed in open-ended glass tubes covered with muslin and returned to the tank for seven days.

Eight animals appeared relatively healthy at the end of the seven-day period and were prepared as described above. The cerebral ganglion was observed to have enhanced yellow fluorescence (Fig. 6). Many more cell bodies (10–11 μ in diameter) emitted the yellow fluorescence. The fluorescence intensity increased so greatly that the individual yellow fluorescing inclusions could not be distinguished. The neuropile region appeared to contain very little green fluorescence. At the beginning of the cerebrovisceral connective many more yellow fluorescing beads could be found. Also, the connective tissue sheath exhibited an enhanced yellow fluorescence.

By contrast, the visceral ganglion in general exhibited an enhanced green fluorescence. In the cortex region, this was due to enhanced green fluorescence from the nerve cell bodies. Due to the high concentration of green fibers in the neuropile, a yellow-green fluorescence was actually obtained. This yellow-green fluorescence was due to the presence of green fibers only, as demonstrated by taking thinner sections. In the neuropile region there was usually a very good supply of varicose fibers which appeared as lightened areas in the green mesh, but in the denervated animal these yellow varicose fibers were greatly reduced in number.

A close look at the transected ends of the cerebrovisceral connective also proved interesting. On the cerebral ganglion side of the transection there was an accumulation of the yellow fluorescing beads and an absence of green fluorescing fibers. On the visceral ganglion side, the connective appeared to contain enhanced green fluorescence, especially at the cut surface (Fig. 7), and no yellow beads. These results were quite dramatic when compared to the cerebrovisceral connective of control animals. This normally contains both green fibers and yellow beads distributed evenly throughout the length of the connective. The distribution

bution of fluorescence throughout the cortex (C), neuropile (N) and sheath (S) with no indication of discrete granules or varicosities. Scale bar = 20 μ.

FIGURE 8. Green fluorescing cells of the cerebral ganglion in an area that contained no yellow fluorescence. The fluorescence is diffuse throughout the whole cell. These cells comprise the fluorescent group in the center of the photomicrograph but many appear relatively isolated from each other. Scale bar = 20 μ.

FIGURE 9. Section through seven-day transected cerebrovisceral connective, showing the accumulation of intensely fluorescing green material on the visceral ganglion side of the cut (arrow). Scale bar = 20 μ.

FIGURE 10. A portion of the cerebral ganglion after two days exposure of whole animals to nialamide in the bathing medium. Cells and fibers show enhanced fluorescent intensity of either yellow or green, and more than the usual number of cells appear to be fluorescent. Scale bar = 20 μ.

of orange pigment with the cerebrovisceral connective seemed unaffected by the transection experiments.

The gills from these animals were also examined but did not appear to have been affected by the transection. Both green and yellow fluorescing elements were present and seemed quite normal in their appearance and distribution.

Pharmacological agents

Two animals were each injected in the posterior adductor muscle and basal portion of the foot with a total of 1 mg of serotonin in a volume of 0.2 ml. They were then placed in a beaker containing 300 ml of artificial sea water in the regular tank for temperature control and continuously aerated.

After two days they were prepared as described above. There was enhanced yellow fluorescence in both ganglia in the nerve cell bodies, in the neuropile, and in the sheath, indicating an ability to incorporate this exogenous supply of serotonin (Fig. 8). The cerebral ganglia had greater fluorescent intensity than the visceral ganglia, which appeared duller yellow. The yellow fluorescence of these cells after loading was homogenous in its intracellular appearance contrary to its normal localization in granules. In the gill axis the branches of the branchial nerve exhibited an enhanced yellow fluorescence but fibers in the individual filaments appeared normal. Where the fibers of the branchial nerve entered the gill filament, the intensity of the fluorescence was normal.

Dopamine was prepared and administered to two animals exactly as in the serotonin experiment. The animals were kept as described above for two days. The results were similar to those described for serotonin, except that the cells, nerve fibers, and sheath emitted greatly enhanced green fluorescence. In thick sections the neuropile of the visceral and cerebral ganglia gave a dull yellow fluorescence as a result of the concentration effect of excess dopamine (Falck and Owman, 1965). The gill filaments, however, tended to have a greater green distribution. The specific green fluorescence was not just confined to the supporting rod as in the normal tissue, but was also seen in the epithelial cells.

Four different animals were each injected with 2 mg of reserpine in 0.1 ml volume and kept for two days as described above. There was depletion of the monoamines as indicated by a decrease in fluorescence intensity. The exact distribution of fluorescence was obscured by a blurring effect, apparently the result of amine release from its storage sites (Fig. 9). This was observed directly and is not simply due to the long exposure time required for photomicrography of depleted tissue. It was difficult to properly discern the color green from the color yellow. The connective tissue sheath increased in fluorescence indicating that it has the ability to pick up the released amines.

The monoamine oxidase inhibitor nialamide was used to enhance intracellular pools of specific monoamine fluorescence. Two animals were bathed in an instant ocean solution that contained 1.3 mg of nialamide per ml solution for two days. In the cerebral ganglia there was an increase in the yellow and green fluorescence. The yellow vesicles in the nerve cell bodies located in the cortex increased both in number and in the intensity of fluorescence. There was also obvious increases in the number of fluorescing cells (Fig. 10). The intensity of green fluorescence of the neuropile was also enhanced.

The effect on the visceral ganglion was less pronounced except in the cortical region where the brightest green fluorescence was obtained. There was also an increase in the concentration of the non-specific orange pigment described earlier.

The gill appeared to have an enhanced but more diffuse green fluorescence. Several yellow fluorescing structures, such as blood cells and granules in the epithelium, also exhibited greater fluorescence. The green supporting rod could be seen in each gill filament but not as clearly as in the normal gill because surrounding tissue also fluoresced green.

Discussion

The foregoing description is in general agreement with those of previous authors regarding the arrangement of cell bodies into groups within the cortex and nerve fibers into tracts within the neuropile (Rawitz, 1887), the presence of distinctly green or yellow fluorescing cells and fibers (Dahl *et al.*, 1966), the predominance of green fluorescence in the visceral ganglion and of yellow fluorescence in the cerebral ganglion (Dahl *et al.*, 1966; Sweeney, 1968), and the absence of any large cells such as those found in many gastropods but not yet found in bivalves.

The specificity of yellow fluorescence from the serotonin fluorophore seems well established (Falck and Owman, 1965) and the histofluorescent localization of this amine in the nervous system and gill of *M. edulis* is in agreement with its detection there by various chemical means (Welsh and Morehead, 1960; Aiello, 1960, 1962). Identification of the green fluorescing fluorophore as that of dopamine rests on several observations. Sweeney (1963) found high concentrations of dopamine but no epinephrine or norepinephrine in the ganglia of *M. edulis* and six other bivalve species and Malanga *et al.* (1972) found an abundance of dopamine but only traces of norepinephrine in extracts of *M. edulis*. In the present experiments, treatment with formaldehyde vapor at 80° C for 3 hours instead of the usual 1 hour did not increase the extent or intensity of fluorescence even though it would have brought out fluorescence due to a secondary amine, such as epinephrine, had it been present (Falck and Owman, 1965). This kind of identification has been made for dopamine in *Anodonta cygnea* (Zs.-Nagy, 1968), *Anodonta piscinalis* (Dahl *et al.*, 1966) and *Spisula solida* (Cottrell, 1968) but not for the green fluorescence in *Sphaerium sulcatum* (Sweeney, 1968) or *Elliptio complanata* (Paparo, 1972), but it is likely that dopamine is the only catecholamine present in physiologically significant amounts in bivalve nervous systems.

The present data clearly establishes the presence of dopaminergic and serotonergic innervation of the gill of *M. edulis* in agreement with the pharmacological findings described in the introduction. Individual green and yellow fibers can be distinguished in the branchial nerve and in some of the small branches which it sends into the gill filaments as it runs anteriorly in the gill axis. Paparo (1972) reported finding only weakly green fluorescing fibers in the gills of *M. edulis* and *E. complanatus*, and Sweeney (1968) reported only yellow fluorescing fibers in the gill filaments of *S. sulcatum*. Paparo (1972) also presented clear electron micrographs of nerve fibers lying in indentations of the ciliated epithelial cells and closely apposed to the underlying supporting rod. In the present work, longi-

tudinal sections revealed the presence of green and yellow fibers in this region but did not show the epithelial cells in the kind of orientation that allowed unequivocal identification as to type. In cross section, specifically fluorescing specks could only be seen in some sections, probably when the section contained a varicosity, and these were predominantly in the anterior portion of the filament where the ciliated cells are located but the exact point of innervation could not be determined.

Experiments with dopamine, serotonin and nialamide indicated that the supporting rod can take up dopamine and opens up the possibility that its normal green fluorescence is due to the absorption of dopamine released from adjacent nerves during preparation of the tissue. The same thing could have occurred with serotonin in Sweeney's work since the yellow fiber he describes is too large in diameter to be the fine fibers seen in electron microscopy. Although endogenous dopamine seems to be confined to the nerves and possibly the supporting rod in *M. edulis*, serotonin is found not only in the nerves but in the eosinophilic blood cells and, if nialamide is used to prevent its oxidation, in the epithelial cells of the gill. The blood cell serotonin responds to reserpine, nialamide, and exogenous serotonin as does the serotonin in the nervous system. Contrary to the report of Paparo (1972) we found no nerve cell bodies in the gill despite extensive searching, and we believe that without careful comparison of morphology, staining characteristics and relation to other cells, these serotonin-containing blood cells, which are wandering amoebocytes and sometimes send out long pseudopods, may be mistaken for nerve cell bodies.

Regarding the role of the serotonergic innervation of the gill we have no evidence for a sensory function as proposed by Sweeney (1968). In *M. edulis* the organism was never observed to respond to stimulation of the gill, we observed no sensory structures in the gill, and the nerves always appear to lie under the epithelial cells and have never been observed to pass up between them toward the surface as might be expected of sensory fibers.

A question that could not be answered from our observations was that concerning the possible presence of non-fluorescent fibers to the gill. There are many non-fluorescent fibers present in the origin of the branchial nerve. Bouffard (1970) showed sections in which all the fibers of the branchial nerve were histochemically positive for acetylcholine esterase. In almost identically appearing sections through the branchial nerve as it innervates individual filaments, we found that all the fibers are histochemically fluorescent. Because of their close packing and very small diameter one must concede the possible presence of non-fluorescent fibers in each small group and these were looked for in the present study by phase contrast microscopy but without success. In view of our present finding that exogenous amines can be taken up by nerves not exhibiting specific fluorescence for their endogenous presence it is possible that all fibers become fluorescent during preparation. The same could apply to the deposition of the cobalt sulfide precipitate in the cholinesterase staining and the problem remains unresolved.

The branchial nerve comes from the visceral ganglion and the present study indicates that dopaminergic and serotonergic cell bodies in that ganglion send fibers into the branchial nerve. The serotonin-containing cells are larger but fewer in number, and this is in keeping with the predominance of green color in the

branchial nerve. Transection of the cerebrovisceral connective decreased the amount of yellow fluorescing granules and varicose fibers in the connective on the visceral ganglion side of the cut, in the neuropile and in the cortex but did not alter the intensity of fluorescence in cell bodies or fibers in the branchial nerve. This indicates that fibers from the cerebral ganglion do not simply pass through the visceral ganglion on their way to the gill but terminate there, apparently on several kinds of cells. Transection of the connective caused an increase in green fluorescence in the ganglion but did not affect the branchial nerve. On the cerebral ganglion side of the cut and in the cerebral ganglion itself, there was a great enhancement of yellow fluorescence. The role of this cerebral-to-visceral-ganglion transport of serotonin has not yet been determined but some preliminary experiments (Catapane, Aiello and Stefano, 1974) suggest that it has to do with stimulating the cilioexcitatory serotonergic fibers originating in the visceral ganglion. Dahl *et al.* (1966) performed a similar experiment in *A. piscinalis* with similar results regarding the increase in serotonin in the cerebral ganglion, cortex and neuropile and its decrease in the visceral neuropile. They did not observe yellow fluorescing cells in the visceral ganglion cortex and since there is no information on the function of the branchial nerve in that species we can not assess the physiological significance of their experiment.

An observation of general interest which we made consistently throughout this study is that in *M. edulis* endogenous, intracellular yellow fluorescence is always in granules whereas green fluorescence always appears to be homogenously distributed. This granular localization of serotonin may be lost following treatment with reserpine, nialamide or exogenous serotonin, suggesting that it is its normal, physiological condition to be so localized. Zs.-Nagy (1968) identified the site of dopamine localization in the cerebral ganglion of *A. cygnea* to be 1000 Angstrom diameter dense core vesicles, which would be below the limit of resolution of the light microscope. He did not comment on the serotonin sites. Dahl *et al.* (1966) mention yellow fluorescing granules but do not comment on the green fluorescing cells. In the absence of specific statements by other authors we do not know how general a condition this is in bivalves but it appears to be consistent in *M. edulis*.

The significance of the orange auto-fluorescing pigment observed by ourselves and others in both monoaminergic and other neurones remains to be clarified (Zs.-Nagy, 1968; Dahl *et al.*, 1966) but we can be sure that in *M. edulis* it is subject to seasonal change. The fact that this pigment is increased by nialamide treatment and that prolonged exposure to UV light slowly converts it to a yellow fluorescing pigment suggests that it might be metabolically related to serotonin. The increase in the number of specific yellow-fluorescing cells following treatment with nialamide might be explained if one assumed that cells which originally contained orange autofluorescent pigment were potentially serotonergic and accumulated serotonin when its oxidation was inhibited by nialamide.

Summary

Monoamine localization was accomplished in *Mytilus edulis* by the use of histofluorescence. Intracellular stores of dopamine and serotonin were found to be

synthesized in the proper neuron and transported down the axon to the terminal varicosities.

Most of the cells in the cortex of the cerebral and visceral ganglia were non-fluorescent. Of the fluorescent cells, serotonin predominated in the cerebral ganglion and dopamine predominated in the visceral ganglion. There was a net flow of serotonin in the cerebro-visceral connective from the cerebral to the visceral ganglion and a net flow of dopamine in the opposite direction.

Serotonin fluorescence was localized in intracellular granules in neurons and blood cells. Dopamine fluorescence was distributed homogenously in neurons and in the supporting rod of the gill. The visceral ganglion supplies the gill with nerve fibers of both types.

Exogenously supplied serotonin and dopamine were taken up by both kinds of nerve cells and by some other tissues. Endogenous stores of both amines were altered in content and distribution by reserpine and by nialamide.

The distribution of monoamine in the nervous system and gill lends further support to the notion of a dual innervation mechanism controlling ciliary activity in the gill.

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